

A chloroplastic inner envelope membrane protease is essential for plant development

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Abstract Regulated intramembrane proteolysis (RIP) is a fundamental mechanism for controlling a wide range of cellular functions. Cleavage of membrane embedded proteins results in soluble fragments exerting their function, e.g., as transcription factors and thereby regulating gene expression. This process is highly conserved throughout all kingdom of life as are the involved proteases. RIP has been described in eukaryotes, bacteria and archaea though until recently not in plant organelles. Here we describe a chloroplastic membrane protease which belongs to the conserved S2P family of membrane metallo proteases. We show that this protease is localized in the inner envelope membrane and is essential for plant development. It could function in a RIP like process regulating the concordant action in the plant cytosol, nucleus and plastids.

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1. Introduction

In the field of intracellular signalling a new concept has emerged during the last few years: cleavage of transmembrane proteins within the plane of the membrane, liberating soluble cytosolic fragments that control gene transcription. This mechanism, called regulated intramembrane proteolysis (RIP), influences processes as diverse as cellular differentiation, lipid metabolism, membrane biogenesis and stress response [1]. RIP proteases can be monomers or complex hetero-oligomers and can belong to the aspartic, metallo- or serine protease families. These proteases are widely conserved and are present in bacteria, archaea, and eukaryotes [2], though until recently none had been found in plant plastids.

Plastids are a heterogeneous family of organelles found ubiquitously in plant and algal cells [3]. Most prominent are the chloroplasts which carry out essential functions such as photosynthesis, the biosynthesis of fatty acid as well as of amino acids.

As mitochondria, chloroplasts derived from a single endosymbiotic event. They are believed to have evolved from an an-

cient cyanobacterium which has been engulfed by an early eukaryotic ancestor [4]. During evolution the plastid genome has been greatly reduced and most of the genes have been transferred to the host nucleus. Consequently, >98% of all plastid proteins are translated in the cytosol and must be post-translationally imported into the organelle. Thus, chloroplast development is dependent on the coordinated expression of both plastid and nuclear encoded genes [5]. Especially import into the chloroplast, which is mediated by complex molecular machines in the outer (Toc complex) and inner (Tic complex) envelope of chloroplasts, respectively [6], and assembly into functional complexes have to be tightly regulated. Many groups work on the signalling network between the nucleus, the cytosol and chloroplasts but conclusive results are still lacking.

Quite recently, a membrane-associated metalloprotease has been described in plant chloroplasts designated Egl1, which seems to be required for plastid development [7]. The authors identified a consensus pentapeptide (HEXXH), typical for zinc-dependent proteases, and another conserved motif (NPDG) which were first found in S2P-like proteases [8]. Though the actual substrate of Egl1 is still unknown it could be demonstrated that the protease is critically involved in thylakoid grana formation as well as in the accumulation of chlorophyll and chlorophyll *a/b* binding proteins in chloroplast membranes.

Egl1 belongs to a family of homologous putative proteases in *Arabidopsis*, one of which is At2g32480. There are several proteases known which exert their function in chloroplasts [9]. Nevertheless, no protease has been described which cleaves its substrate within the membrane. So it is not possible to assort Egl1 or its homologues to any of the known plastidic protease families except for it being dependent on zinc as a cofactor. The closest orthologue of At2g32480 in bacteria is RseP (YaeL) from *Escherichia coli*. RseP has been shown to cleave the anti-sigma factor RseA in conjunction with the bacterial stress response [10]. YaeL is located in an operon, adjacent to YaeT which intriguingly is homologous to the chloroplastic import channel of the outer envelope membrane, Toc75 [11]. YaeT has been shown to be involved in β -barrel protein transport in the bacterial outer membrane [12]. Because of the homology of YaeT to Toc75 we strived to determine if the putative protease coded for by At2g32480, which we tentatively named AraSP, might be involved in the cleavage of pToc75 during import. Toc75 has a very unusual feature for outer membrane proteins: it contains a bi-partite transit peptide. Upon import the first part is cleaved off by

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the stromal processing peptidase [13] whereas the protein responsible for the second processing (iToc75 to mToc75) had not been identified at the onset of our studies. So we decided to investigate expression and localization of AraSP. Our biochemical analysis demonstrated that AraSP is localized in the chloroplastic inner envelope membrane. Investigation of antisense and knockout mutants of *Arabidopsis* led to the conclusion that this protease is essential for plastid development. However, our experiments indicated that AraSP has a different substrate than iToc75. This was corroborated by a paper from Inoue et al. [14] in which the authors convincingly describe Psp11 as the protease responsible for processing iToc75. Therefore, other possible substrates for AraSP are discussed.

2. Methods

2.1. Transcription and translation

The coding regions for the proteins of interest were cloned into vectors under the control of the SP6 or T7 promoters. All constructs were controlled by DNA sequencing. Transcription was performed in the presence of SP6 or T7 RNA polymerase and the resulting mRNA was translated in a reticulocyte lysate system (FlexiSystem, Promega, Madison, USA) in the presence of [³⁵S] methionine [15] at 25 °C for 45 min. The translation mixture was then centrifuged at 250 000 × g for 10 min at 4 °C and the post-ribosomal supernatant was used for all import studies.

2.2. Chloroplast isolation from pea and protein import

Chloroplast isolation and import experiments were conducted as described previously [15]. In some cases chloroplasts were treated with 6 M urea after import to separate bound pre-proteins from membrane-integrated polypeptides. Urea (6 M) treatment in 50 mM HEPES-KOH, pH 7.6, was carried out for 15 min at 25 °C. Insoluble proteins were collected by centrifugation at 250 000 × g for 10 min.

2.3. Isolation of intact chloroplasts from *Arabidopsis thaliana*

Arabidopsis chloroplasts were isolated from 3–4-week-old plants grown in a climate chamber at 20 °C. All procedures were carried out at 4 °C. The leaves were grinded in isolation buffer (50 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.33 M sorbitol, 14.3 mM β-mercaptoethanol) and the suspension was filtered through four layers of cheese cloth. Chloroplasts were collected after centrifugation at 1000 × g for 3 min and purified on 40–80% Percoll gradients by centrifugation for 15 min at 3000 × g in a swing out rotor. Intact chloroplasts were collected from the 80% interface, washed twice with isolation buffer and centrifuged at 1000 × g for 10 min.

2.4. Fractionation of isolated chloroplasts from *Arabidopsis thaliana*

Chloroplasts were prepared as described above. Chloroplasts were ruptured by hypo-osmotic treatment in 20 mM HEPES, pH 7.6. The suspension was centrifuged at 6000 × g for 20 min to sediment thylakoid pellets. The supernatant containing stroma and chloroplast outer and inner membranes was centrifuged at 250 000 × g for 1 h, to pellet the mixed outer and inner chloroplastic membranes. Thylakoids and the mixed membranes were resuspended in phosphate buffer (10 mM Na₃PO₄) and used in further experiments.

2.5. Generation of antisense mutant

The N-terminal 525 bps of At2g32480 were amplified by PCR and cloned in the binary vector pGTPV. This resulted in an antisense construct under control of the 35S-promoter which was then transformed into chemically competent *Agrobacterium*. These were then used to infect *Arabidopsis* plants [16]. Plants were selected by phosphinotricine (BASTA) and resistant ones were cultivated until flowering. Seeds were harvested and selected on BASTA containing agar plates. After transfer on soil plants were further selected with BASTA.

2.6. Analysis of T-DNA insertion lines from *Arabidopsis thaliana*

T-DNA lines for At2g32480 and At1g05140 were ordered from GABI KAT (Cologne). The insertions were verified by PCR on genomic DNA with one gene specific and one T-DNA specific primer. The same method was used for screening for homozygous mutants in both plant lines.

2.7. Purification of antibodies

The polyclonal antiserum raised against amino acids 222–337 of AraSP was affinity purified by coupling the antigen on CNBr activated sepharose and incubating the serum with this matrix. Bound antibodies were eluted with 0.2 M glycine, pH 2.5, and immediately buffered in 0.1 M Tris/HCl, pH 8.0.

3. Results and discussion

3.1. AraSP belongs to a conserved metallo protease family

At2g32480 belongs to the conserved family of membrane metallo proteases. This family is characterized by a distinct metal binding motif HEXXH and an additional highly conserved motive NPDG. These motives have been first described by Rudner et al. [8] for the SREBP S2P protease involved in the mammalian sterol biosynthesis. Fig. 1 demonstrates that the *Arabidopsis* genome contains at least three members of the protease family, namely At1g05140, At2g32480 and At5g35220 (Fig. 1A). The first two share about 98% identity which indicates that they might have originated from gene duplication.

For those proteases of the S2P family already partly characterized it was predicted by secondary structure prediction that the active centre comprising the zinc binding site HEXXH lies either within the membrane or very close to the surface of the bilayer. For full length AraSP (At2g32480) TMPred predicts four to five strong transmembrane alpha helices (Fig. 1B). The HEXXH motif is localized directly between the first two helices and should therefore be very close to the membrane surface. In addition AraSP contains a PDZ-domain known to be involved in protein–protein interaction [17]. According to computational models, the active side as well as the PDZ domain are facing the inner, i.e., stromal side of the membrane (Fig. 1B). RseP as well comprises a PDZ-domain which was shown to interact with DegS, another protease in the signalling cascade [18].

Affymetrix data analysis (<http://arabidopsis.info>) revealed that At2g32480 is expressed in nearly all developmental stages of *Arabidopsis*, though expression is highest in green seedlings and cotyledons whereas it is very low in roots and certain stages of siliques and seeds (Fig. 1C).

3.2. AraSP is localized in the inner envelope membrane of chloroplasts

According to ChloroP, a program predicting the probability of a protein being imported into plastids [19], AraSP is a chloroplast protein with an N-terminal transit peptide of 73 amino acids. To corroborate these theoretical results a cDNA coding for AraSP was used to synthesize the protein in rabbit reticulocyte lysate and in vitro import assays with isolated pea chloroplasts were conducted (Fig. 2A + B). We could show that AraSP is indeed imported into chloroplasts in a time-dependent manner and then processed to the mature form of about 41 kDa. Treatment of chloroplast membranes with 4 M urea after import indicated that AraSP becomes largely

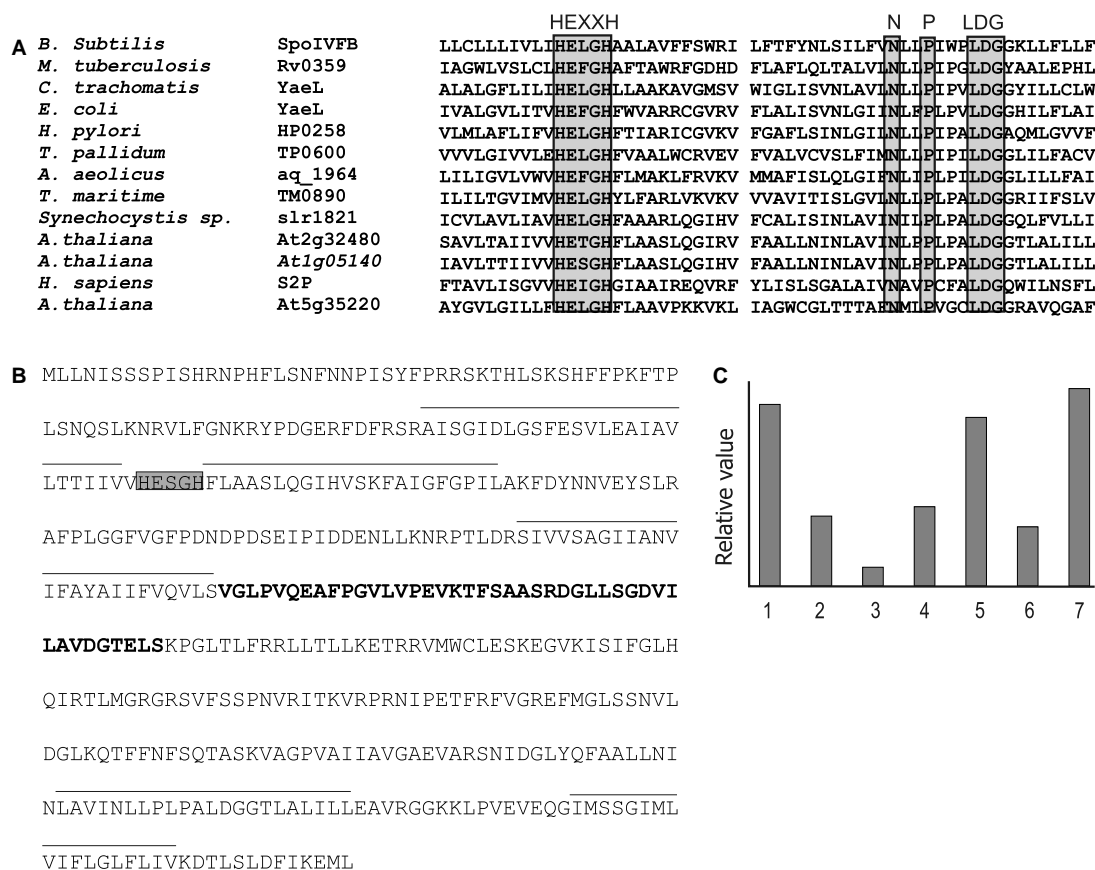


Fig. 1. Primary and secondary structure analysis of AraSP. (A) Alignment of S2P-like proteases from different organisms. The conserved motifs HEXXH and NPDG are highlighted in grey. (B) Secondary structure prediction for the full length AraSP (At2g32480) according to TMPred. Predicted transmembrane helices are indicated by an upper line, the HEXXH active site is shaded in grey and the PDZ domain is in bold letters. (C) Affymetrix analysis (<http://arabidopsis.info>) reveals differential expression of At2g32480. 1–7: 7-day-old plants grown on soil under continuous light. 1: Cotyledons, 2: hypocotyl, 3: roots, 4: shoot apex + vegetative + young leaves, 5: leaves 1 + 2, 6: shoot apex, vegetative, 7: seedling, green parts.

embedded into the membrane during the course of import. Urea is known to extract proteins that are only associated with or partly embedded into membranes [20]. These results are in agreement with the secondary structure prediction.

Fractionation of chloroplasts after import into envelope membranes, thylakoids and stromal fraction consistently showed localization of [35S] labelled matured AraSP in the envelopes (Fig. 2B). The band detectable in the stroma might correspond to a soluble import intermediate which can also be seen in Fig. 2A after up to 10 min of import. The slightly different running behaviour compared to the mature form in the envelope fraction is due to the high amount of large subunit of Rubisco in the stroma which “squeezes” AraSP.

To further investigate the subcellular localization we raised an antibody against a polypeptide comprising amino acid 222–337 of the pre-protein and used it for immuno-blotting of chloroplast compartments. Fig. 2C shows further evidence that AraSP is an integral component of plastid envelope membranes as shown by probing different chloroplast subfractions and resistance to urea extraction (Fig. 2C). AraSP is not exclusively found in the pellet as after import, but a significant portion of the protein is in the urea soluble membrane fraction. The reason for the slightly divergent results is most likely the higher concentration of urea used in this setup compared to the import experiment. In order to get a more precise localiza-

tion of the protease we fractionated the envelope membranes into outer and inner membranes. Fig. 2D shows an immuno-blot of outer (OE) and inner (IE) envelope, thylakoids (T) and stroma (S) with affinity purified antibody against AraSP. The protease is exclusively detected in the inner envelope membrane fraction. As a control we probed the upper part of the membrane with antibodies against Toc75 and Tic110, respectively. Toc75 is mainly found in the outer envelope, whereas Tic110 resides in the inner envelope. We conclude that AraSP is located in the inner envelope of chloroplasts. This demonstrates the presence of a putative RIP protease in plastids.

To investigate the effect of light on expression of AraSP *Arabidopsis* wild type seeds were germinated separately in the light or in the dark. Total protein extracts from 14-day-old seedlings from each group were used for immuno-blotting. AraSP can only be detected in light grown seedlings (Fig. 2E).

3.3. AraSP plays an essential role in plant development

To explore the role of the AraSP metalloprotease in plant development we made antisense plants (Section 2). After selection we isolated 18 independent plant lines from which we collected the seeds and tested germination on MS plates. About 50 seeds of all 18 lines were germinated on MS medium. The average percentage of germination was 40%. Three groups of mutant seeds could be distinguished (Fig. 3). Some looked like

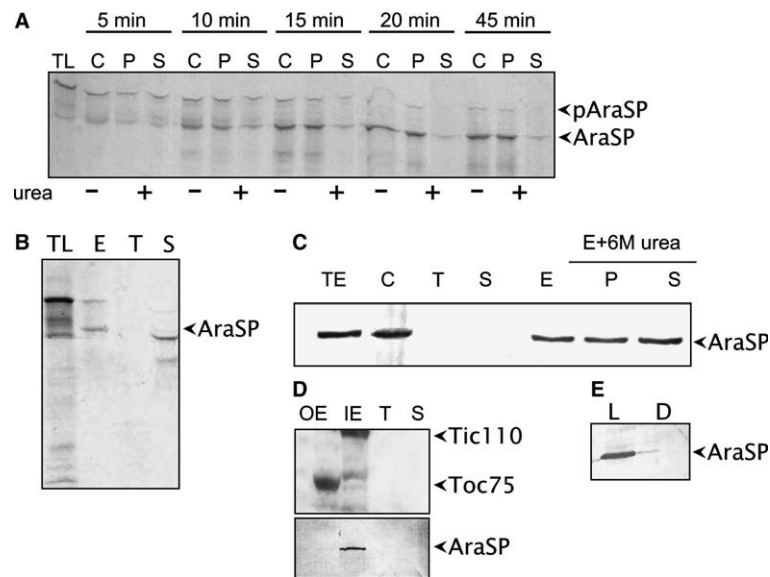


Fig. 2. AraSP is a light regulated integral membrane protein of plastids. (A) S^{35} labelled translation product of AraSP (TL) has been incubated with isolated pea chloroplasts for the indicated time frames. Half of the probe was then treated with 4 M urea and subsequently separated into pellets (P) and supernatants (S). As a control untreated chloroplasts were loaded (C). p: precursor; m: mature protein. A radiograph is shown. (B) Chloroplasts were subfractionated after import into envelopes (E), thylakoids (T) and stroma (S). A radiograph is shown. (C) Total extract was purified from wild type *Arabidopsis* leaves (TE). Chloroplasts were isolated (C) and one portion was further fractionated into thylakoids (T), stroma (S) and envelope membrane (E). Envelopes were also treated with 6 M urea and separated into pellets (P) and supernatants (S). A Western blot with antibodies against AraSP is depicted. (D) Outer and inner envelope, thylakoids and stroma from pea chloroplasts were loaded in equal amounts (15 μ g protein/lane). The gel was blotted onto nitrocellulose and the membrane decorated with affinity purified antibody against AraSP, Toc75 or Tic110, respectively. (E) Seedlings were grown in the light or dark, respectively. Whole leaf extract was isolated after 14 days of growth and expression of AraSP was investigated by Western blot analysis with α -AraSP.

wild type and grew normally even if transferred on soil after 10 days. Others were significantly smaller with reddish cotyledons and poorly developed roots (Fig. 3, as2). These needed about three weeks to recover and were finally able to grow on soil if transferred after “greening”. The third group of plants (Fig. 3, as1) were very small, had tiny red cotyledons, underdeveloped roots and no apical meristem. They did not survive for more than 20 days. The red colour of the antisense seedlings indicates that they suffer from light stress. This might be due to impaired chloroplast development and/or failure to accumulate the light absorbing pigment system.

To follow up on this observation we investigated chloroplast ultra structure from 14-day-old wild type plants and seedlings of line 13. Fig. 3B shows chloroplasts from wild type and as1 plantlets. Wild type plastids are oval to bean shaped and contain a well-developed thylakoidal system (Fig. 3B, left panel). In contrast, chloroplasts from antisense plants are rounded and have only residual thylakoids which are unstructured and disarranged (Fig. 3B, right panel). These results demonstrate the essential role of AraSP in chloroplast development.

To determine the relative amount of the AraSP protein in wild type compared to antisense plants from the as1 type total protein extracts were prepared and probed by immuno-blotting with α -AraSP. Fig. 3C reveals that the amount of AraSP in the as1 mutant seedlings is generally lower than in wild type plants but differs significantly in the different antisense lines. Considering the severe phenotype of antisense plants it seems that the level of AraSP protein must not drop under a critical level, otherwise it leads to the observed drastic effects in chloroplast development. Since many seeds did not germinate AraSP might actually be essential for plant development.

During the course of our study a T-DNA insertion line of AraSP became available at GABI Kat. The T-DNA insertion is situated in the C-terminal part of At2g32480 (Fig. 3D) and was authenticated by PCR (data not shown). We screened about 300 seedlings germinated on MS plates for the homozygous insertion but could not find any. Approximately one third of the plants were wild type, two thirds were heterozygous. This indicates that the gene coding for AraSP cannot be disrupted without interfering with plant viability. We transferred some of the plants to soil and were intrigued when we observed that even heterozygous plants exhibited a growth phenotype (Fig. 3E). The heterozygous plants were slow in growth as well as flower development and failed to produce healthy siliques. They did not show the severe developmental defect of the antisense plants albeit all parts of those plants were smaller than the WT. This is most likely due to the still functional allele in the heterozygous plants which might render more gene product compared to the antisense plants. The T-DNA analysis confirms our results of the antisense approach, namely that maintaining a certain threshold of AraSP protein amount is fundamental for normal plant development.

To address this further we ordered T-DNA insertion lines not only for AraSP but also for a second gene, At1g01540, which is highly similar to AraSP (Fig. 1A). The T-DNA insertion line of At1g01540 splits according to mendelian rules into 1/4 wild type, 1/4 homozygous and 1/2 heterozygous plants and showed no obvious phenotype, indicating that the protein coded for by At1g01540 is differentially expressed – or not at all – or cannot compensate the loss of AraSP. The gene on chromosome one is still intact in knock-out plants from AraSP as confirmed by PCR on genomic DNA (data not shown). Due

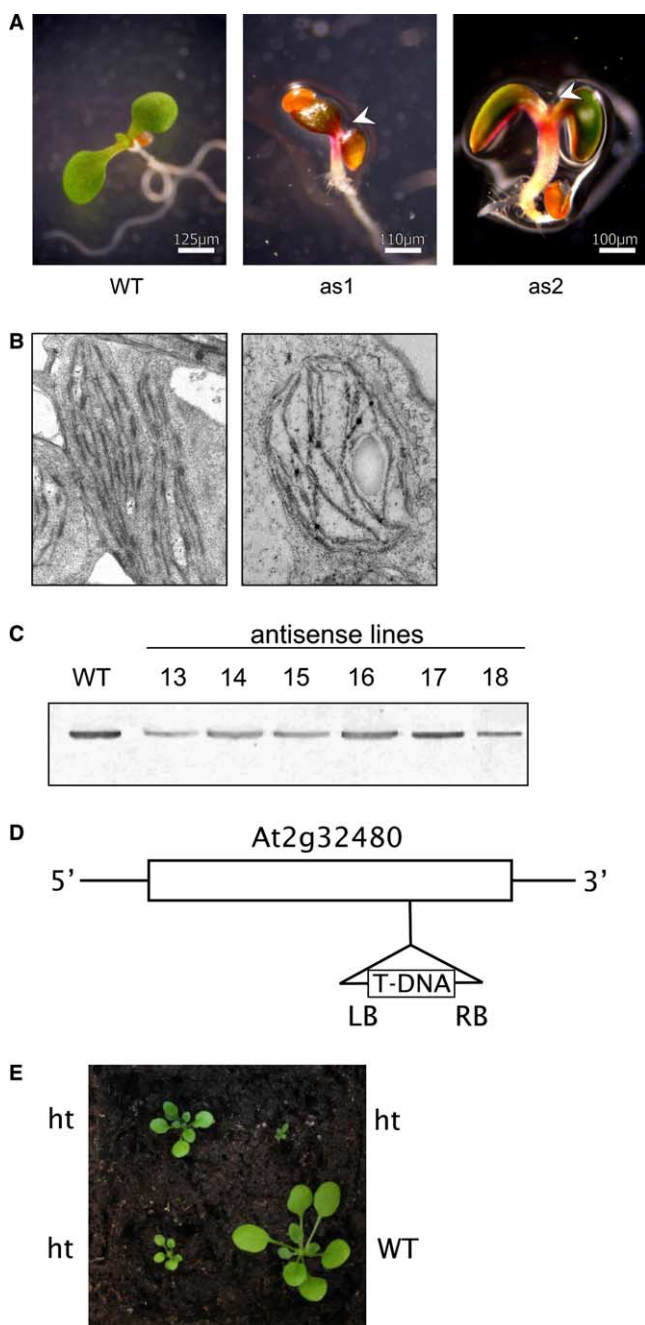


Fig. 3. AraSP is essential for chloroplast development. (A) Seeds from wild type and antisense plants were germinated under the same conditions on MS medium for 14 days. As1 represents the most severe phenotype without apical bud, as2 the intermediate phenotype with reddish cotyledons but with apical meristem. Scale bars for WT: 125 μm; as1: 100 μm; as2: 80 μm. (B) Leaf sections from 14-day-old seedlings from WT and antisense plants were prepared and studied in the electron microscope. Only mutant seedlings with the severe phenotype were used. For WT chloroplasts the magnification was 6500×, for the mutant 8000×. (C) Total protein extract from 14-day-old wild type and mutant seedlings from different antisense lines were prepared and analyzed by Western blot with α-AraSP. Every line contains protein corresponding to the same amount of plant material. (D) Schematic figure of the T-DNA insertion in At2g32480. (E) Phenotype of plants heterozygous for the T-DNA insertion compared to a WT plant. Seedlings were germinated on MS plates and transferred to soil after 14 days. Pictures were taken after 3 weeks growth on soil.

to the high similarity of the two genes it is not possible to distinguish between them in affymetrix expression analysis. This similarity might also lead to co-silencing of the two genes in the antisense approach. However, when we checked the mRNA level in the antisense plants by quantitative PCR with primers that could bind to both genes we got similar results for WT and antisense mutants (data not depicted). This rather indicates that the second gene is still expressed in plants containing the antisense construct against AraSP and the severe phenotype is exclusively due to the lack of AraSP.

Unfortunately, we could not express AraSP in bacteria in an active form; either it was insoluble and inactive or soluble and self degrading, which we were unable to control. We tried several other approaches of testing the protease activity but always ended up with either no or unspecific processing activity. This will have to be dealt with in future experiments.

Another feasible possibility when searching for substrates of AraSP derives from the bacterial systems, where transcription factors are activated upon cleavage by the AraSP homologue RseP. *Arabidopsis* has six different but highly homologous sigma factors which have been assorted to plastids [21]. By Western blotting with polyclonal antisera Mache and colleagues localized especially Sig2 in the soluble compartment of chloroplasts [22]. In contrast, our import experiments show that the mature form of Sig2 is embedded in the membrane like AraSP (data not shown). The plastidic sigma factors are responsible for regulation of the plastid encoded RNA polymerase [23]. Sig2 knock-out plants are only slightly retarded in growth and have pale green leaves but obviously the other sigma factors are able to supersede Sig2. If Sig2 is indeed a substrate of AraSP it is unlikely the only one, because even antisense plants show such a severe phenotype. The exact role of AraSP in chloroplast biogenesis remains to be clarified.

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